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THE UNIVERSITY OF ALBERTA

A STUDY OF AVIAN KALLIKREIN WITH RESPECT TO THE SALT GLAND AND PANCREAS OF THE DOMESTIC GOOSE

by

© SHEILAGH MARTIN

A THESIS

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled "A Study of Avian Kallikrein with Respect to the Salt Gland and Pancreas of the Domestic Goose" submitted by Sheilagh Martin in partial fulfillment of the requirements for the degree of Master of Science.

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[illegible]

Date 16th May 1973

Abstract

A situation exists in goose salt gland that is analogous to one already noted in mammalian salivary glands, namely that stimulation of the secretory nerve causes secretion, which is atropine sensitive, and vasodilatation, which is atropine resistant. There is the possibility that true vasodilator fibers or a metabolic substance (eg. bradykinin) could be responsible for this vasodilatation.

The goose salt gland was studied to determine the presence and possible role of ornithokallikrein. The literature relating to the structure and function of the salt gland was reviewed, as well as that of kallikrein in general and ornithokallikrein in particular.

Aqueous, acetone-aqueous and 75% acetone tissue extracts were prepared from goose and hen pancreas and goose salt gland and were tested biologically and biochemically for ornithokallikrein activity.

Biological assays of goose pancreas were performed using an isolated strip of guinea-pig ileum, which yielded negative results, and an isolated strip of goose ileum, which gave dubious results. In vivo assays, using hen and goose blood pressure, of various extracts showed hypotensive activity. This activity was not inhibited by soyabean trypsin inhibitor in some assays but was inhibited in other assays.

Biological assays of hen pancreatic extracts were performed to check the assay system and provide a basis for comparison with goose extracts. These extracts showed hypotensive activity which was inhibited

by soyabean trypsin inhibitor.

Biochemical assays of both extracts using the synthetic arginine ester, α-N-benzoyl-L-arginine ethyl ester, and selective blocking with soyabean trypsin inhibitor and Trasylol, indicated that ornithokallikrein activity was present.

In general the results obtained, with the exception of inhibition by soyabean trypsin inhibitor in some of the biological assays, were in agreement with previous investigators.

Biological and biochemical assays of goose salt gland extracts, performed under similar conditions showed no activity indicative of ornithokallikrein. Some attempt was made to explain the atypical results of one assay. The possibility that the assay system is not suitable for certain preparations was considered.

Histological observations of the organization of the non-functional salt gland showed that it contained inter- and intralobular connective tissue and in general was similar to those species previously studied.

It seems evident from the experiments that the goose salt gland contains no kallikrein, or if it is present, it cannot be extracted or activated by conventional means.

It was concluded that the ornithokallikrein-kinin system was not responsible for the vasodilatation accompanying secretion, induced by stimulation of the secretory nerve in the goose salt gland.

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I. Introduction

Kallikreins and Kinins:

The investigation of kallikrein began in 1925 when it was shown that a fall in dog arterial pressure could be brought about by injection of human urine. Studies of this substance, first called F-substance, showed it to be a large molecular, non-dialysable, thermolabile substance (Frey, 1926). Further work showed that a similar substance existed in blood (Frey & Kraut, 1928), pancreas (Kraut, Frey & Werle, 1930) and salivary glands (Werle & von Roden, 1936). Frey and co-workers named this new substance "kallikrein" (Greek, kallikreas - pancreas), because they believed that the kallikrein found in all tissues was derived from the pancreas and circulated in the blood. It is now known that kallikreins from different sources are not identical molecules, but do have properties in common.

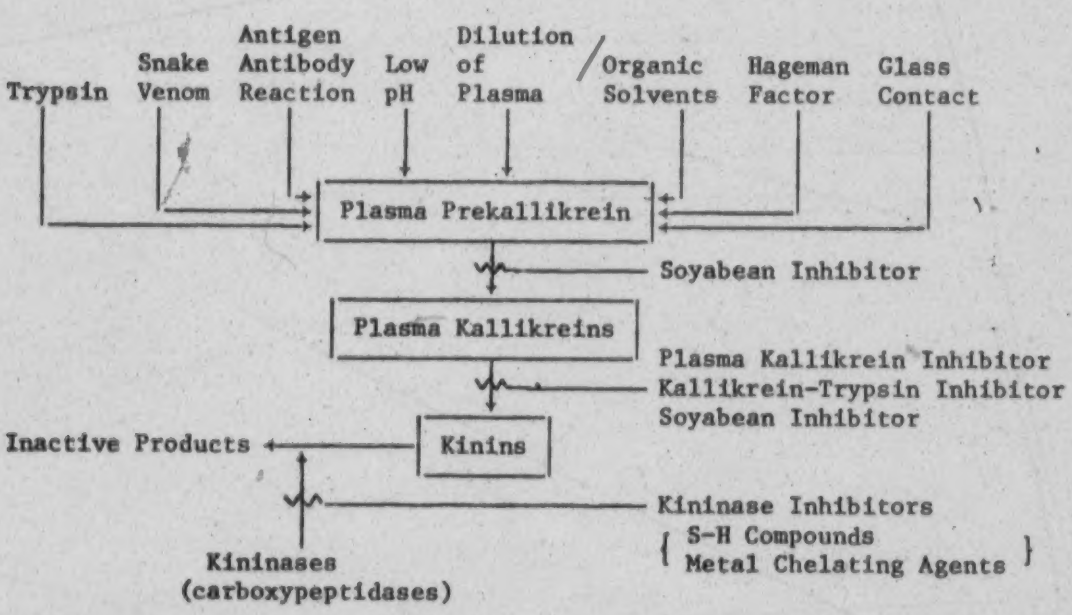
Kallikrein occurs in an active form in salivary glands, saliva and urine and in an inactive (but activatable) form in kidney, pancreas and blood. Chemical investigations have shown that more than one kallikrein exists in hog pancreas (Fritz, Eckert & Werle, 1967) and human plasma (Webster, 1968).

The kallikreins from pancreas, urine and submaxillary gland release kallidin from a specific substrate (kininogen), an α_2 globulin found in plasma or lymph, by a highly specific proteolytic action. They also possess an esterase activity which can be shown by their action on synthetic arginine esters. The molecular weights, ranging from 33,000 to 36,000, and the amino acid composition of these kallikreins are quite

similar. In contrast, plasma kallikreins with a molecular weight of 97,000 have an unspecific proteolytic activity; they release bradykinin instead of kallidin. Among other substances which act like kallikreins are trypsin and snake venoms (Rocha e Silva, Beraldo & Rosenfeld, 1949).

The avian kallikrein-kinin system appears to differ in some respects from that found in mammalian species and will be discussed later in the Introduction.

Kinins (kallidin, bradykinin) are the active peptides released as a result of kallikrein activity. General properties include hypotensive activity, ability to contract smooth muscle (except for relaxation of the rat duodenum), ability to increase vascular permeability and produce pain when applied to a blister base (Schachter, 1969). The following diagram (Schachter, 1969) illustrates the possible path of kinin release in plasma.



A number of pathological and physiological roles have been ascribed to kallikreins and kinins. Although most of these lack sufficient supporting evidence, the kallikrein-kinin system has been implicated in two pathological conditions, carcinoid syndrome and hereditary angioedema (Schachter, 1969). A definite physiological role has not been ascertained for the kallikreins but there has been speculation as to their possible function as blood flow regulators, e.g., glandular functional hyperemia or as a mediator of some vascular changes which occur at birth (Melmon, Cline, Hughes & Nies, 1968).

The possible role of kinins, rather than true vasodilator fibers, as the cause of functional vasodilatation in the submaxillary gland has been argued for some years. The first evidence for vasodilator nerves to the submaxillary gland of the dog was presented by Claude Bernard (1858). Another fact of importance which dealt with chemical transmission of nervous effects was established by Heidenhain (1872). He observed that the vasodilator effect of chorda stimulation was not blocked by atropine whereas the accompanying secretory activity was abolished. Although some reasonable explanations have been put forth to explain this atropine-resistant phenomenon, none has been accepted with final certitude (Ambache, 1955).

The hypothesis that vasodilator, atropine-resistant fibers to the gland exist was uncontested until the twentieth century. Barcroft and Muller (1912) postulated the view that vasodilatation in the submaxillary gland was determined by the secretory metabolism of the gland, but no chemical agent was shown to be responsible for this specific action. Following the discovery of salivary kallikrein by Werle and von Roden (1936), it was suggested that this potent vaso-

dilator was the specific chemical mediator (Ungar & Parrot, 1936). The fact that kallikrein vasodilatation, like chorda vasodilatation, was atropine-resistant, supported this view. The work of Hilton and Lewis (1955, 1956) has extended the view that kinins are responsible for functional hyperemia in salivary glands. Ample evidence has been produced by other investigators to refute this theory and to argue successfully for the existence of true vasodilator fibers (Bhoola, Morley, Schachter & Smaje, 1965; Beilenson, Schachter & Smaje, 1968). Ultrastructural evidence also concurs with the view that vasodilator fibers to blood vessels of the salivary glands do exist (Garrett, 1966a, 1966b).

Recent investigations have attempted to show that vasodilatation in the salivary gland may be initiated by vasodilator fibers and supported and maintained by kinins formed in the activated gland (Gautvik, 1970). Kinins have also been implicated in the after-dilatation which follows a period of sympathetic nerve stimulation (Gautvik, Kriz & Lund-Larsen, 1972).

Statement of Problem:

Research on salt gland secretion and blood flow in the domestic goose has revealed a situation analogous to that existing in the mammalian salivary glands, i.e., when the salt gland is stimulated (osmotic load, nervous stimulation, parasympathomimetic drugs) two events occur - secretion and vasodilatation. Moreover, it was found that secretion but not vasodilatation was blocked by atropine. Reference was made to the fact that two views are held regarding the control of functional vasodilatation: (i) atropine-resistant cholinergic

nerves elicit vasodilatation, and (ii) induced by substances (eg., bradykinin) formed as a result of increased metabolism of secretory cells (Hanwell, Linzell & Peaker, 1971a).

It was then decided to pursue this problem by determining the possible existence of kallikrein in the goose salt gland and its role or lack of it in functional vasodilatation.

The avian nasal (salt) glands are crescent-shaped structures which are located in shallow depressions above the eye orbit. They vary in size, the largest being found in marine species.

Anatomically, each gland consists of two lobes with two ducts which join before reaching the external nares, but are so similar that they are considered as one functional unit (Marples, 1932). Histological studies have shown that lateral branchings from the main ducts form the central canals, while radial outgrowths from each central canal give rise to branched secretory tubules with closed terminal ends. These secretory tubules are lined by simple cuboidal-columnar epithelium and form a lobule which is surrounded by a thick layer of connective tissue (interlobular connective tissue). In addition, each secretory tubule is invested with a thin sheath of connective tissue (peritubular connective tissue). The central canals are embedded in a relatively thick intralobular tissue sheath (Ernst & Ellis, 1969).

The vascularization of the salt glands of gulls and ducks, as well as other species, has been studied (Marples, 1932; Schmidt-Nielsen, Jorgensen & Osaki, 1958) and it has been found that arrangement of blood vessels is very similar in the different species. The main arterial supply is contributed by the arteria ophthalmica interna. This vessel penetrates the orbital wall above the optic nerve and,

passing upwards along the medial wall of the orbit, it divides into two branches to the salt gland. Several small arteries are given off to the gland from the anterior arterial branch, which then continues to the beak. The posterior glandular portion is supplied by the posterior arterial branch. Another artery from the posterior wall of the orbit anastomoses with the posterior branch and contributes to the glandular blood supply. Both arteria ophthalmica interna and arteria ophthalmica externa are branches of the arteria carotis interna. Veins from the salt glands follow the arrangement of arterial vessels.

The connective tissue between individual glandular lobes contains many branching arteries and veins. At intervals, branches from the arteries pass into the lobes. After arteries have reached the central connective tissue they branch into many capillaries. Since the capillaries, for most of their course, run parallel to the secretory tubules, one is reminded of a countercurrent system. However, the strict analogy does not hold because a countercurrent mechanism indicates a single tube looped back upon itself (Schmidt-Nielsen, 1965). One report (Fange et al., 1958a) did not find any lymph vessels draining the salt glands, but their evidence was not sufficiently conclusive to show they do not exist for this particular organ.

Salt gland innervation of ducks, gulls, geese and other species has been the topic of a few studies (Marples, 1932; Fange, Schmidt-Nielsen & Robinson, 1958b; Ash, Pearce & Silver, 1966, 1969; Linzell, McLean & Peaker, 1972), and as with blood vessels, the arrangement of nerves is similar in different species. The gland (as indicated in Figure 1) is innervated by a cholinergic secretory nerve (perhaps a branch of the VIIth cranial nerve) which emerges from

the sphenopalatine ganglion and enters the orbit to become associated with the ophthalmic division of the trigeminal nerve. The ganglion sends fibers into the anterior part of the gland.

Although this ganglion is closely applied to the ophthalmic division of the Vth nerve, it does not appear to have any connections with it (Cottle & Pearce, 1970). Histochemical studies have shown that stained elements run between the columns of secretory cells and also along the walls of the ducts. At the periphery of the lobules the staining was often associated with a series of looped structures, the distribution of which was similar to but not identical with the blood vessel arrangement (Ash et al., 1969). It is possible that the secretory nerve contains somatic afferent nerves and sympathetic post-ganglionic nerves, the latter probably including vasomotor fibers to glandular blood vessels (Fange, Krog & Reite, 1963; Ash et al., 1969). There is also the possibility that the secretory cells receive sympathetic innervation (Linzell et al., 1972).

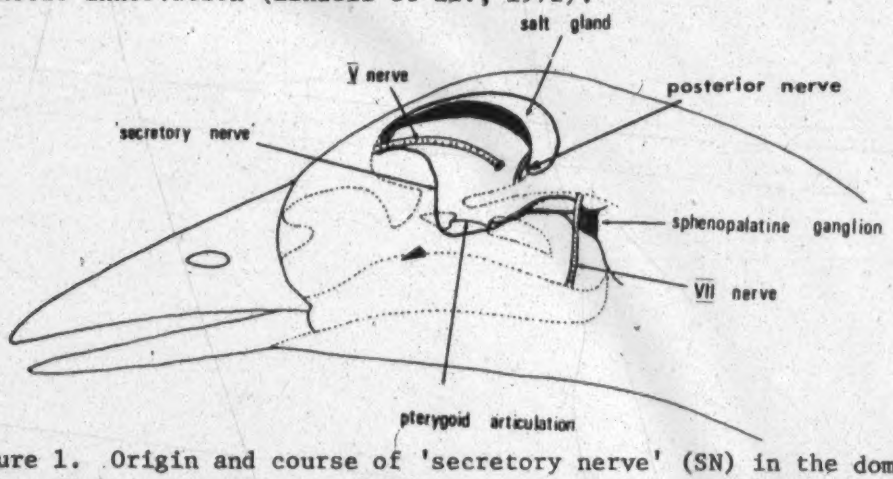


Figure 1. Origin and course of 'secretory nerve' (SN) in the domestic duck. Within the orbit the SN is bound to the edge of the Harderian gland before separating from it to meet the Vth nerve (ophthalmic division); at this junction the ganglionic enlargements are found in the SN. The cervical sympathetic trunk gives rise to a branch depicted entering the sphenopalatine ganglion from below (Ash, Pearce & Silver, 1969).

The presence of the nasal glands was first described by Nicholaus Steno in 1664 (see Fange et al., 1958a). Early workers had thought that their role, through elaboration of a secretion, was to protect the nasal passages from the effects of sea water. Their true functional significance, as extrarenal excretory organs, was clearly demonstrated by Schmidt-Nielsen, Jorgensen and Osaki (1957).

Generally, these glands are largest and functional in marine or estuary-dwelling birds, but may be made functional in such species as ducks and geese (Ellis, Goertemiller, De Lellis & Kablotsky, 1963; Hanwell, Linzell & Peaker, 1970).

A number of studies have shown that salt gland secretion is initiated in response to sea water or an osmotic load, such as mannitol or hypertonic NaCl (Fange et al., 1958; Schmidt-Nielsen, 1960; Ash, 1966; Hanwell et al., 1971a), and is maintained, as in mammalian salivary glands (see Burgen & Emmelin, 1961), by neural stimulation which is believed to be cholinergic (Fange et al., 1958; Fawcett, 1962; Ash et al., 1969; Hanwell et al., 1971a; Hanwell, Linzell & Peaker, 1972).

The secretory response is probably initiated by receptors which detect an increase in plasma tonicity (Ash et al., 1969; Hanwell, Linzell & Peaker, 1971b; Hanwell et al., 1972; Stewart, 1972) as first suggested by Schmidt-Nielsen, Jorgensen and Osaki (1958), rather than responding to an expansion of blood volume as suggested by other investigators (Holmes, 1965; Burford & Bond, 1968). The location of these receptors had been thought to be in the head, either in the CNS (Schmidt-Nielsen, 1960) or in the ganglion supplying the cholinergic fibers to the gland (Ash et al., 1969). A more recent study has indi-

cated that these receptors lie in the heart (Hanwell et al., 1972).

Investigations have indicated a varying sensitivity of osmoreceptors in different species. The goose is particularly sensitive to increased plasma tonicity and will invariably respond to so-called isotonic (0.154M) NaCl. It has been found that plasma Na and Cl concentrations were increased after the injection of large volumes of 0.154M NaCl (Hanwell et al., 1971b). Since no western gull and only a few ducks respond to this stimulus, osmoreceptors in the goose are considered most sensitive (Hajjar, Sattler, Anderson & Gwinup 1970; Peaker, Peaker, Hanwell & Linzell, 1972). Since denervation (Ash et al., 1969) and cross-circulation studies (see Peaker, 1971b) have shown that secretion is dependent on intact innervation, there is no evidence to support the view that hormones elicit normal secretion (Phillips, Holmes & Butler, 1961).

Only one study of denervation sensitivity has been reported (Gill & Burford, 1968). These workers found that chronically-denervated salt glands were supersensitive to an oral salt load and that injection of parasympathomimetic drugs demonstrated the classic denervation sensitivity in terms of decreased threshold and increased duration of effect. However, they did not indicate which nerves were cut in their experiments.

The rate of secretion varies among the different species. Gulls have been found to secrete up to .6 ml/g tissue/min (Schmidt-Nielsen, 1960), geese up to 1.9 ml/g tissue/min (Peaker, 1971b) and ducks .186 ml/min (Ash et al., 1969). The composition of the nasal fluid is predominantly Na and Cl ions, in about equal concentrations and with a small K ion concentration. Ducks and geese vary from 400 -

700 mEq Na^+/l, and gulls from 700 - 800 mEq Na^+/l. One type of gull has been found to secrete up to 1100 mEq Na^+/l (Schmidt-Nielsen, 1960). No other components, eg., protein, mucin, Mg^{++} ions and NH_4^+ ions have been found in the secretion (Scothorne, 1959a).

Salt loading a bird initiates a number of changes in the salt gland. One of the most important is increased enzyme activity, particularly ouabain-sensitive Na^+-K^+-ATPase (Ernst, Goertemiller & Ellis, 1967; Fletcher, Stainer & Holmes, 1967). Gland size increases (Benson & Phillips, 1964; Ballantyne & Fourman, 1966) and as well a rise in glandular RNA has been observed (Ballantyne & Wood, 1969). It is of interest to note that the pituitary prolactin content of ducks is increased by 50% of the second day of salt feeding, and declines to subnormal levels by the fifth day (Ensor & Phillips, 1970). It has also been found that prolactin affects the secretory rate of ducks given a minimal stimulatory load of NaCl. Since the increased secretory rate could not be related to increases in plasma osmolality, it is thought that prolactin may directly affect the salt gland (Peaker & Phillips, 1969; Peaker, Phillips & Wright, 1970).

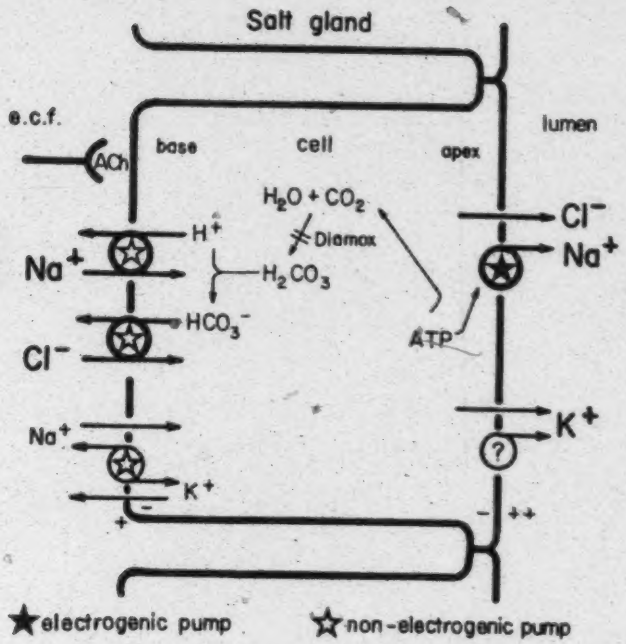
Early ultrastructural studies on the glands of marine birds (Doyle, 1960) showed that the basal but not the luminal membrane of principal (secretory) cells was greatly infolded. In a thorough study of salt-stressed ducklings by Ernst and Ellis (1969), similar structural changes were evident. The peripheral cells which lie at the blind end of the secretory tubules are the same in both control and experimental ducklings. The partially-specialized secretory cells which are a transitional type, have fairly elaborate infoldings of the lateral but not the basal membrane. The fully specialized secretory cells are

packed with mitochondria and have numerous infoldings of basal and lateral membranes. This increase in surface area allows for greater absorption. The apical membrane does not show any infolding.

A number of investigators have looked at the question of the establishment of a concentration gradient between plasma and secreted fluid. A study of the intracellular concentration of Na^+, K^+ and Cl^- ions in vivo and in vitro (Peaker, 1971a), showed that the concentration of Na^+ and Cl^- ions was lower in the gland than in plasma. This finding was in agreement with previous workers and therefore it might be concluded that the concentration gradient is established across the luminal membrane during secretion.

There is evidence to suggest the presence of a Na^+ pump on the luminal membrane of the secretory cell. An electrophysiological study of the salt gland has shown that during secretion the gland duct is electrically positive to the blood by 40 to 60 mV (Thesleff & Schmidt-Nielsen, 1962). Both secretion and potential difference were abolished by ouabain administration. Large quantities of ouabain-sensitive Na^+/K^+ ATPase have been found in the gland (Hokin, 1963; Ernst et al., 1967) and one study (Ballantyne & Wood, 1970) has shown that Na^+/K^+ ATPase is concentrated on the luminal side of the cell.

The proposed Na^+ pump (Figure 2) operates electrogenically with Cl^- following passively. Since the K^+ ions appearing in the secretion are not reaccumulated, it is thought that extrusion of Na^+ ions is not coupled with K^+ influx (Peaker, 1971b). However, K^+ ions are necessary for stimulation of the pump (von Rossum, 1966).



e.c.f. extracellular fluid

Figure 2. A possible mechanism to account for ion transport in the salt gland (Peaker, 1971b).

The ion movements across the basal membrane do not present such a clear picture. The role of ACH in stimulating salt transport is not known, but it appears likely that it may have several stimulatory effects on the secretory cell rather than a single, primary action. Whether it acts directly or via an intracellular messenger has not been established.

Although evidence gathered thus far presents varying views, it seems quite plausible that a non-electrogenic pump transporting Na^+ and Cl^- into the cell might be operative at the basal membrane during secretion. This would involve movement of Na^+ and Cl^- into the cell in exchange for H^+ and HCO_3^-. This hypothesis (Peaker, 1971b) is supported

by the work of earlier investigators (Fange et al., 1958; Nechay, Larimer & Maren, 1960).

During high secretion rates, the salt gland can remove large quantities of salt from the arterial plasma. In order to supply the salt glands with water, ions and metabolic substances, the blood flow through these glands must be high. Although vasodilatation had been observed indirectly during secretion (Fange et al., 1958b; Thesleff & Schmidt-Nielsen, 1962; Fange et al., 1963), it was difficult to obtain any quantitative measurements due to the inaccessibility of the blood supply.

A comprehensive study of salt gland blood flow (Hanwell et al., 1971a), which combined the indicator fractionation technique of Sapirstein (1956, 1958) with the thermodilution method for cardiac output (Fegler, 1954), allowed for accurate measurement of changes in blood flow during secretion. In general, the results showed that in the control gland the flow was comparable to mammalian salivary glands, but in the salt-stressed geese this flow was increased about 14-fold.

Since the rate of blood flow is not related to secretion rate, the calculated extraction of ions and water from plasma was variable. However, in one goose, 57% Na and 80% Cl were removed from the plasma and secreted. It is probable that the large surface area of the in-folded basal membrane in salt-stressed birds could be partly responsible for the large uptake from the extracellular fluid.

Ornithokallikrein:

A number of studies have been undertaken to show the presence and role of various enzymes in functional and non-functional salt glands:

acetylcholine esterase (Ballantyne & Fourman, 1966; Ash et al., 1969; Cottle & Pearce, 1970), acid phosphatase (Scothorne, 1960; Ellis et al., 1963; Kühnel, Petry & Burock, 1969), acyltransferase (Levine, Higgins & Barnett, 1972), alkaline phosphatase (Scothorne, 1960; Ellis et al., 1963), (Na^+-K^+)-adenosine triphosphatase (Ernst et al., 1967; Ballantyne & Wood, 1968; Ernst, 1972), butyrylcholine esterase (Ballantyne & Fourman, 1966; Ash et al., 1969; Smith, Fourman & Haase, 1971), carbonic anhydrase (Schmidt-Nielsen, 1960), cytochrome oxidase (Ellis et al., 1963), α-β-galactosidase (Kühnel et al., 1969), β-glucuronidase (Ballantyne & Wood, 1967; Kühnel et al., 1969), monoamine oxidase (Ellis et al., 1963), succinic dehydrogenase (Scothorne, 1959b; Ellis et al., 1963). However, no work has been done on possible kallikrein content. To date, the major research has been done on pancreatic kallikrein which has been found in goose, hen, duck and pigeon and was named ornithokallikrein (Werle & Hurter, 1936). As in mammals, no kallikrein was found in the liver or muscle of chicken and goose (Werle & Hurter, 1936), but was found in goose, chicken and pigeon feces.

This ornithokallikrein, which cleaves the arginine ester α-N-benzoyl-L-arginine ethyl ester (BAEe), was found to be hypotensive in birds only, inhibited by bovine pancreatic, hen and cattle plasma inhibitors, as well as Trasylol, but was not affected by soyabean trypsin inhibitor (Werle, Hochstrasser & Trautschold, 1966).

The action of ornithokallikrein on avian plasma releases an ornithokinin which is hypotensive only in birds, is rapidly inactivated by hen plasma and contracts the chicken ileum, a tissue insensitive to mammalian kinins (Werle et al., 1966). Acidification of plasma (pre-incubated at pH 2 for a short time and then held at pH 7.5) will produce

the polypeptide (Werle et al., 1966; Werle & Leysath, 1967). There is no definite evidence indicating that the kinins produced by ornitho-kallikrein and acidification of plasma are the same. However, they both have a molecular weight of 6,000 to 6,500 and the amino acid composition of the "spontaneously-produced" kinin has been determined (Werle & Leysath, 1967). The following table indicates what residues are present.

Table I. Amino acid composition of ornithokinin.

Amino Acid	Residues	Amino Acid	Residues
Arginine	8	Proline	4
Lysine	6	Serine	4
*Histidine	2	*Alanine	2
*Glutamic Acid	2	Glycine	2
*Aspartic Acid	2	*Threonine	2
Isoleucine	4	Tyrosine	2
*Leucine	4	*Valine	2
Phenylalanine	4		

The fact that seven of the above amino acids (*) are not found in other kinins, plus other evidence already cited, indicates that ornithokinin is quite different from kinins found in other species.

A study of the plasma kinins of reptilian and avian plasmas has revealed some interesting facts. If glass beads are rotated in reptilian or mammalian blood and exposed to avian plasma, kinin production is initiated. Thus, a prekallikrein, kininogen and kininase but not a contact factor are present. This released kinin contracted

the rat uterus and guinea-pig ileum and lowered the perfusion pressure in a dog's hind limb. Carboxypeptidase B also inactivated this kinin (Erdos, Miwa & Graham, 1967). Acidification of duck plasma by a method similar to that performed by Werle et al. (1966) released a kinin which, when assayed against bradykinin, was more active on guinea-pig ileum or dog hind limb perfusion than on rat uterus. Whether this kinin is similar to that released by activation with reptilian contact factor remains to be finalized. Definitely they are different from ornithokinin because it has no effect in mammalian systems. This evidence raises the possibility that birds have two kinin systems, one which lacks the contact factor, and the second releases ornithokinin which is inactive in mammals (Erdos et al., 1967).

General Summary:

With the information provided by the literature review as background, the following procedures were used in the study of avian kallikrein and the histological organization of the goose salt gland.

1. Preparation of suitable aqueous and/or acetone extracts from goose salt gland and pancreas.
2. Preparation of suitable extracts from hen pancreas for purposes of:
   (a) checking the experimental technique;
   (b) comparison with goose extracts.
3. Biological assays were performed by:
   (a) using isolated strips of guinea-pig, hen and goose ileums to measure the kinin released from a suitable substrate;
   (b) measuring the vasodilatation induced by kinin released

from plasma in the intact hen and goose.

4. Biochemical assays were performed using the synthetic substrate α-N-benzoyl-L-arginine ethyl ester (BAEe).

5. Determination of salt gland organization was ascertained by a brief histological study.

II. Materials and Methods

Animals:

Fourteen domestic geese (4.3 kg - 6.6 kg) of either sex were used in the experiments. Of these, five were used for biological assays and nine were used to obtain salt and pancreatic glands. Generally, birds were exsanguinated and tissue removed.

Eleven chickens (1.75 kg - 3.15 kg) of either sex were used for experimental procedures. Of these, three were used for biological assays and eight were sacrificed to obtain pancreatic tissue.

Anesthetic:

Birds were anesthetized with sodium pentobarbital (25 mg/kg I.V.). One goose was anesthetized with 60 mg/kg I.P., but this method was found to be too slow and was discontinued.

Surgical Procedures and Recordings:

Intravenous injections (drugs, anesthetic) were made through a cannula in the brachial vein. A tracheal cannula was inserted. Arterial blood pressure was recorded from the carotid artery via a Statham pressure transducer connected to a Grass pen recorder.

Preparation of Tissue Extracts:

A. Goose Salt Gland:

1. Aqueous Extracts: Two pairs of glands were excised from birds which had been especially sacrificed or used for biological assay. The

glands were cleared of connective tissue, washed in saline, weighed and freeze dried. These were then homogenized with distilled water to which a drop of 1% Triton had been added and the resulting homogenate was centrifuged at 10,000 rpm and 4°C for 20 minutes. The supernatant was freeze dried and stored in a dessicator.

2. Salt gland extracts were also prepared by a combination acetone-aqueous method. The excised glands were washed in saline, weighed, freeze dried and homogenized in 50% acetone (2 ml/g wet wt.). The resulting homogenate was centrifuged at 10,000 rpm and 4°C for 30 minutes. The resulting precipitate was dried overnight in a dessicator under partial vacuum. An aqueous extract was made and the suspension centrifuged under the conditions already noted. The supernatant was dialysed overnight at 4°C against a large volume of distilled water. The resulting clear solution was freeze dried and stored in a dessicator.

3. (a) Another aqueous extract of the gland was prepared by the following method. One pair of glands was excised, cleared of connective tissue, distilled water added and pH adjusted to 4.5 with hydrochloric acid. The preparation was left 4 1/2 hours at room temperature so that activation could occur. The pH was then adjusted to 6.7 after addition of distilled water. The tissue was homogenized and centrifuged at 10,000 rpm and 4°C for 20 minutes. The supernatant was removed and used for biochemical assay.

3. (b) Some tissue was prepared as above with one additional step. The supernatant recovered after centrifugation was dialysed overnight at 4°C against distilled water.

4. (a) Aqueous extracts were also prepared by homogenizing the tissue with an equal volume of distilled water, centrifuging the homogenate at 13,000 rpm and 4°C for 30 minutes and dialysing the supernatant overnight at 4°C against distilled water. Some of the supernatant was used for biochemical assay and the remainder was freeze dried. On one occasion the precipitate from centrifugation after dialysis was kept and freeze dried with the supernatant.

4. (b) Acetone extracts of this freeze dried material were prepared by adding 30% acetone (2 ml/g wet wt.) and homogenizing. The homogenate was centrifuged at 10,000 rpm and 4°C for 15 minutes, and to the resulting supernatant was added pure acetone to a final concentration of 75% (2.6 ml/ml supernatant). The suspension was centrifuged at 10,000 rpm and 4°C for 15 minutes and the precipitate dried in a dessicator under partial vacuum.

B. Goose Pancreas:

1. Goose pancreatic extracts were prepared as in (2) above.

2. Acidified extracts of glandular tissue were prepared as in (3) above but biochemical assays failed to indicate any kallikrein activity. It was found that the dialysis step (which followed the preparation of the aqueous extract) seemed more important than acidification, so most pancreatic extracts were prepared as in 4(a) and 4(b) above.

C. Hen Pancreas:

1. Acidified tissue extracts were prepared as previously described.

2. It was found that dialysis of the aqueous extracts and subsequent preparation of acetone precipitates yielded the best assay results. Tissues were handled as described in 4(a) and 4(b) above. A summary of the different tissues and modes of preparation is found in Table II.

Table II. Tissue Extracts.

Animal	Tissue	Preparation	Method
Goose	Salt Gland	Aqueous Extract	1
		Acetone (50%)-Aqueous Extract	2
		Acidified Aqueous Extract	3
		Aqueous-Acetone (75%) Extract	4
Goose	Pancreas	Acetone (50%)-Aqueous Extract	2
		Acidified Aqueous Extract	3
		Aqueous-Acetone (75%) Extract	4
Hen	Pancreas	Acidified Aqueous Extract	3
		Aqueous-Acetone (75%) Extract	4

Preparation of Substrate:

Attempts were made to measure the potency of kallikrein solutions (gland extracts) by assaying the amount of kinin that might be released from a substrate. Substrates used were fresh dog plasma, 'heated dog globulin' and 'heated chicken and goose globulin'. The preparation of 'heated substrate' entailed defibrination, heating plasma

to destroy any kininase, and dialysis against saline. After precipitation at 4°C with $(NH_4)_2SO_4$, saturated at 0°C, the suspension was centrifuged. The resulting precipitate was dissolved in water (1/3 original plasma volume) and dialysed at 4°C for 48 hours. The supernatant collected after centrifugation was freeze dried (Holdstock, Mathias & Schachter, 1957).

Conditions of Assay:

Biological assay of kallikrein activity was performed by: 1. Using the guinea-pig, goose and hen ileum to measure kinin released from dog, goose or hen plasma. The assay preparation, a piece of isolated ileum, was suspended in a 10 ml bath in Tyrode solution (mM/1: NaCl, 34.25 of 4M stock; KCl, 2.7 of 1M stock; $CaCl_2$, 1.8 of 1M stock; $MgCl_2$, .5 of 1M stock; NaH_2PO_4, 3.6 of 1M stock; $NaHCO_3$, 24 of 1M stock; glucose, 1 g/1), at 30° - 32°C or 37°C (hen) and aerated with 95% O_2-CO_2 mixture. The load on the ileum was one or two grams. Contractions of the ileum were recorded with a Grass force transducer which was connected to a Grass pen recorder. 2. Measuring the vasodilatation induced by kinin released from plasma in the intact animal. The pressure recording was made from the carotid artery as previously described.

Biochemical Assay:

Biochemical assay of kallikrein activity may be carried out with ease and high reproducibility by using synthetic substrates. Kallikrein has a high specificity of the proteolytic activity, but is less specific as an esterase, cleaving only arginine esters such as α-N-benzoyl-L-arginine methyl ester (BAMe), α-N-benzoyl-L-arginine ethyl

ester (BAEe) and α-N-p-tosyl-L-arginine methyl ester (TAMe). Hydrolysis of BAEe (found to be the best substrate) to benzoyl-L-arginine and ethanol allows five different methods for assaying esterolytic activity. The method found most satisfactory was spectrophotometric determination of kallikrein activity by enzymic ethanol assay. With this method a change of absorbance of 0.0011 ΔE/min/3 ml total test volume in every NAD-dependent reaction corresponds to an enzyme activity of one milli-unit. About 5 mU or .1 KU of kallikrein can be determined with this method. The assay as described by Trautschold (1970) was used.

Assay:

Wavelength 366 nm; total volume cells 1.5 ml; temperature 25°C; incubation time 5 - 10 minutes. 1.0 ml pyrophosphate (0.2M) - semicarbazide (0.2M) - glycine (0.05M) buffer (pH 8.5) was placed in the cell; 0.05 ml of nicotine amide dinucleotide (NAD, 0.03M), 0.1 ml of alcohol dehydrogenase suspension (30 mg enzyme protein/ml) and 0.25 ml of α-N-benzoyl-L-arginine ethyl ester HCL (BAEe, .006M) were added, mixed and incubated for 5 minutes. The reaction was started by adding 0.1 ml or less of the kininogenase. The zero optical density was recorded against distilled water and readings were taken every minute for 10 minutes. Increase of absorbance per minute should not exceed 0.03.

Standard kallikrein (1 mg/ml) and bovine pancreatic trypsin (4 mg/25 ml .001N HCl) were also used with BAEe and the efficacy of the inhibitors, soyabean trypsin inhibitor (SBTI, 3 mg/1.5 ml buffer) and Trasylol (5,000 KIU/ml) were tested against these preparations. Trasylol is a kallikrein-trypsin inhibitor of bovine origin. Although

benzoyl arginine-p-nitroanilide HCl (BANA) is the best substrate for trypsin (Nagel, Willig, Peschke & Schmidt, 1965; Haverback, Dyce, Bundy & Edmundson, 1960; Trautschold, 1970), problems in obtaining adequate results precluded the use of this substrate. Because of this difficulty, BAEe was used for determination of kallikrein and trypsin activity and the use of suitable inhibitors, SBTI and Trasylol, has enabled an elucidation of separate activities.

Protein Analysis:

The soluble protein content of the aqueous and acetone extracts was determined by the assay method described by Lowry, Rosebrough, Farr and Randall (1951), with some modification.

Assay:

Wavelength 660 nm; total volume of cells 3 ml; temperature 25°C. 20 ml of solution A (2% Na_2CO_3 in .1N NaOH), 0.2 ml solution B (1% $CuSO_4$) and 0.2 ml solution C (2% sodium potassium tartrate) were mixed. The sample (tissue extract) was added to 1 ml H_2O, 5 ml of (A+B+C) were added and incubated for 10 minutes. 0.5 ml phenol reagent (1:1 dilution) was added and incubated for 10 minutes. The sample was read against a blank containing all of the above except the experimental sample. Protein content was determined by reading from the standard curve.

Preparation of Histological Specimens:

Small pieces of salt gland were excised as quickly as possible and fixed for at least 12 hours in Bouin's solution (75 ml saturated

aqueous solution of picric acid; 25 ml 10% formalin; 5 ml glacial acetic acid). The specimens were washed for 24 hours in running water, dehydrated with alcohol, cleared with xylene and embedded in paraffin. Sections (7μ) were cut with a steel knife on a Leitz-Wetzlar rotary microtome and stained with Harris' haematoxylin and eosin. Sections were mounted in a synthetic resin, Permount, and examined with a Zeiss light microscope.

Drugs:

acetylcholine chloride; atropine sulfate; bradykinin; heparin sodium; histamine; mepyramine maleate.

III. Results

Physiological Results

Goose Salt Gland:

Biological Results: An aqueous extract of goose salt gland assayed on guinea-pig ileum gave negative results. As illustrated in Figure 3, a dose of ACh (0.05 µg) indicated that the gut was sensitive but no kinin-like activity was shown in assays involving salt gland extract, salt gland and goose plasma substrate, and incubation of a mammalian substrate, fresh dog plasma and salt gland extract. Generally the gut is sensitive to bradykinin at a concentration of 1 to 10 ng/ml.

The possible hypotensive effect of an ornithokallikrein-ornithokinin system was assayed in three geese. Aqueous preparations of the salt gland (2 mg; 8 mg) failed to elicit any vasodilatation as indicated in Figure 4A. The work of previous investigators (Werle et al., 1966) indicated that mammalian kinins have no effect on avian systems. A test with bradykinin was in agreement with this result.

Acetone extracts (method 2) of varying concentrations (100 µg; 750 µg; 2.2 mg) tested in two geese failed to elicit any response. In contrast, acetone extracts of goose pancreas did have a hypotensive effect, indicating the possible presence of ornithokallikrein (Figure 4B).

Figure 5 shows the result of salt gland extract (method 4) tested in one chicken. The intravenous injection of a 5 mg sample did not elicit any response, while the same concentration of goose pancreatic extract and hen pancreatic extract (100 µg) caused a vasodilatation.

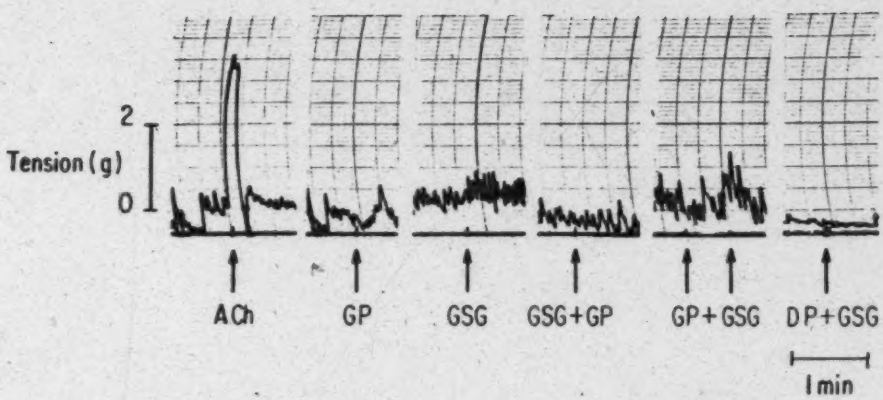


Figure 3. Bioassay of goose salt gland extract on guinea-pig ileum.

1) acetylcholine, 0.05 µg
2) goose plasma (50 mg/ml), 0.2 ml
3) goose salt gland, 1 mg
4) goose salt gland (1 mg) and goose plasma substrate (0.2 ml) incubated 1' 37°C
5) goose plasma substrate (0.4 ml) and goose salt gland (1 mg)
6) fresh dog plasma substrate (0.3 ml) and goose salt gland (1 mg) incubated 1' 37°C

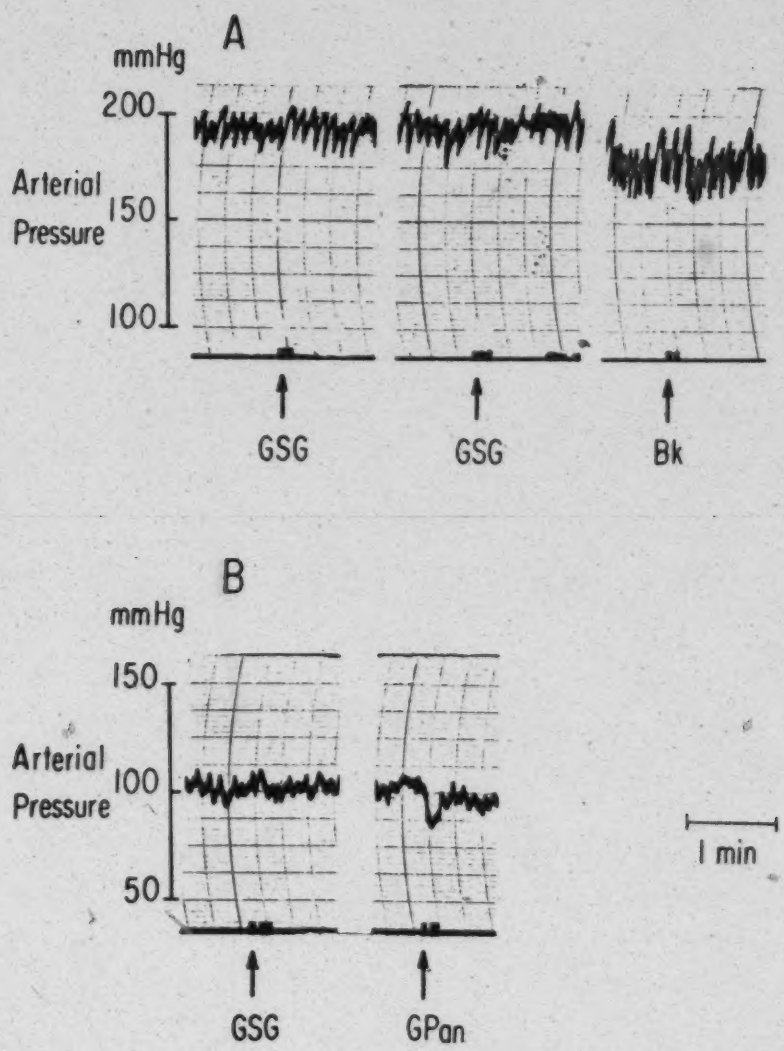


Figure 4. Bioassay of goose salt gland extract on blood pressure of two geese.

A. 1) goose salt gland, aqueous extract, 2 mg
   2) goose salt gland, aqueous extract, 8 mg
   3) bradykinin, 1 µg

B. 1) goose salt gland, acetone extract, 2.2 mg
   2) goose pancreas, acetone extract, 2.2 mg

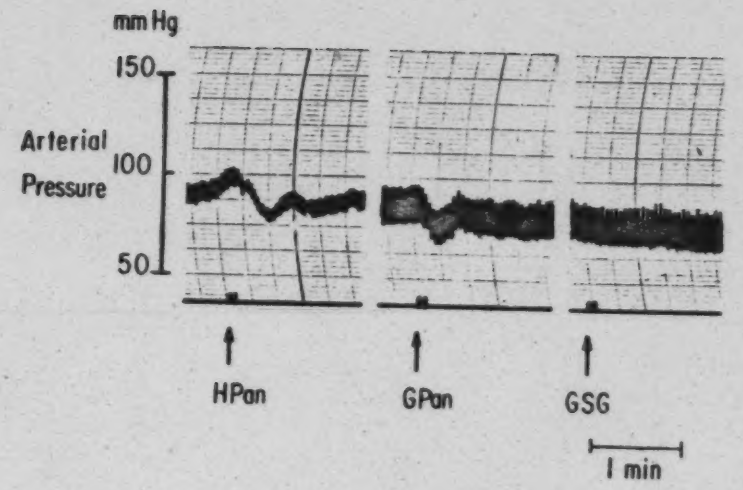


Figure 5. Bioassay of goose salt gland extract on hen blood pressure.

1) hen pancreas, 100 µg
2) goose pancreas, 5 mg
3) goose salt gland, 5 mg

(all extracts are 75% acetone precipitates).

Goose salt gland extracts were not assayed on the isolated goose or hen ileum.

Biochemical Results: Prior to performing the assays of the pancreatic and salt gland extracts, the inhibitors, SBTI and Trasylol, were checked against standard kallikrein and trypsin. The general pattern indicating the rate of activity is shown in Figure 12.

Two attempts to measure kallikrein activity were made without any success. The first assay utilized an acidified, undialysed aqueous preparation (method 3a) and the second assay was done on an acidified, dialysed aqueous extract (method 3b). Although this assay showed non-specific esterase activity, it was not possible to distinguish any activity indicative of kallikrein.

A third assay was done using a 75% acetone preparation (method 4) and unusual results occurred. There was some indication of non-specific esterase activity, but the use of inhibitors gave results which showed that kallikrein-like activity was present (.98 KU/mg protein; 2.4 KU/g fresh tissue). It was not possible to reconcile this finding with other biological and biochemical data. The pattern of activity showed an apparent potentiation of activity by SBTI.

No ornithokallikrein activity was detected in any of the biological assays performed (goose and hen blood pressure). Biochemical assays, with the one exception, also yielded negative results.

Goose Pancreas:

Biological Results: The biological assay using the guinea-pig ileum and goose pancreatic acetone extract (method 2) failed to elicit any response. As shown in Figure 6, goose plasma and dog plasma were used as substrates.

One assay using the isolated goose ileum (Figure 7) gave dubious results. The gut was responsive to ACh but not to mammalian bradykinin. Samples tested were goose plasma, trypsin and goose pancreas extract, using goose plasma as substrate. Since it was difficult to maintain a steady base line, it cannot be said with certainty whether the slight contraction which appeared with goose pancreas and plasma was due to released kinin or irregular natural activity.

Many in vivo assays of goose pancreatic extracts on three geese yielded interesting results: A threshold dose of pancreatic extract was difficult to ascertain as intraspecies variation was indicated by the fact that varying concentrations (250 µg to 2.5 mg) had to be used to elicit a response. In order to separate trypsin and ornitho-kallikrein activity, the inhibitor, SBTI, was used. In tests on two geese, similar concentrations of trypsin and pancreatic acetone extract (method 2) were incubated with SBTI. As illustrated in Figure 8, the trypsin was blocked but there was no effect on the vaso-dilatation evoked by the pancreatic extract. However, a reversal of these findings was obtained in two tests on one goose. Figure 9 shows that goose pancreatic extract (method 4) elicited a hypotensive effect which was definitely blocked by SBTI used in the same ratio

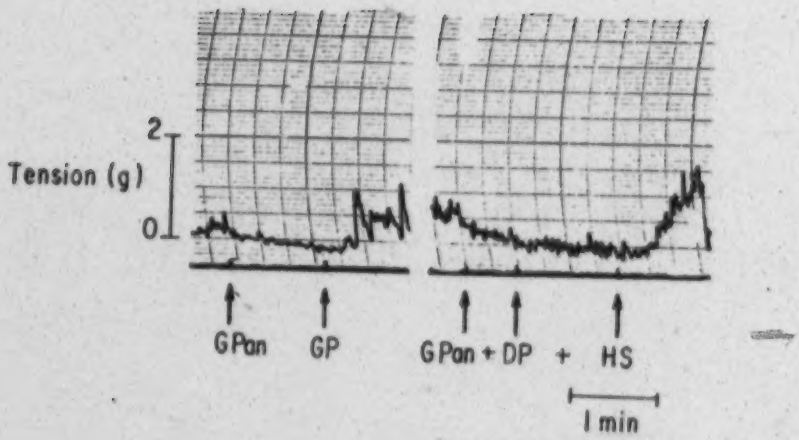


Figure 6. Bioassay of goose pancreas extract on guinea-pig ileum.

1) goose pancreas, 5 mg, and goose plasma substrate (50 mg/ml), 0.4 ml
2) goose pancreas, 5 mg, + fresh dog plasma, 0.3 ml + human saliva, 0.1 ml

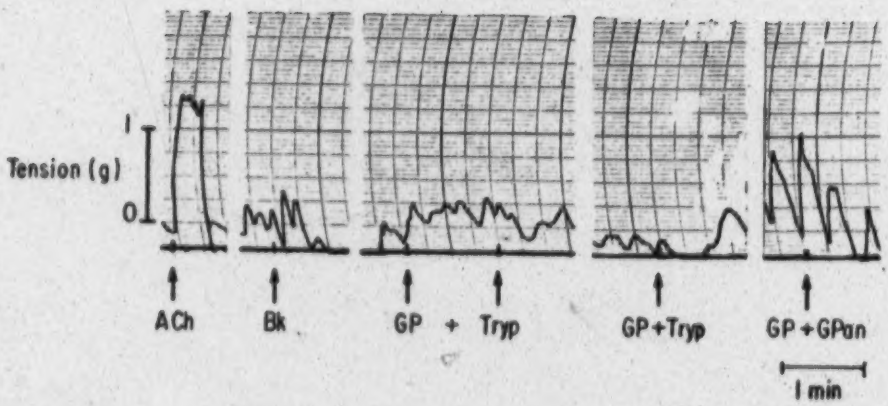


Figure 7. Bioassay of goose pancreas extract on goose ileum.

1) acetylcholine, 1 µg
2) bradykinin, 0.5 µg
3) goose plasma substrate (15 mg/ml), 0.3 ml and trypsin, 0.1 mg, not incubated
4) goose plasma substrate, 0.3 ml, and trypsin, 0.1 mg, incubated 1' 37°C
5) goose plasma substrate, 0.3 ml, and goose pancreas, 0.5 mg, incubated 1' 37°C

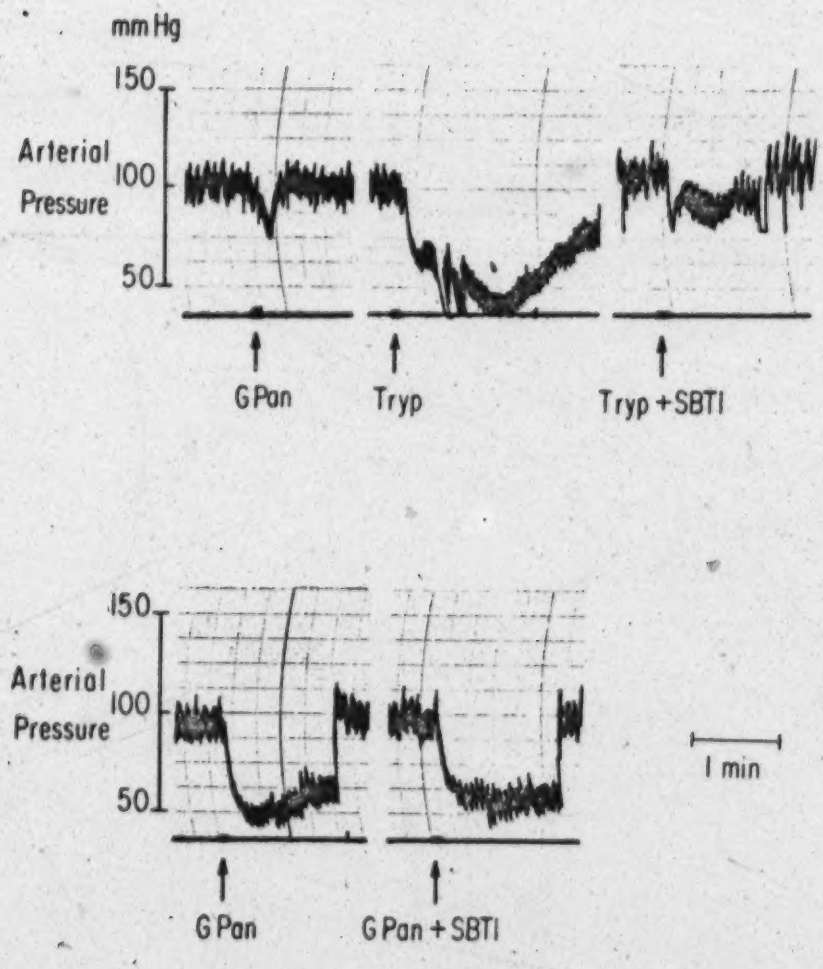


Figure 8. Bioassay of goose pancreas extract on goose blood pressure.

1) goose pancreas, 250 µg
2) trypsin, 5 mg
3) trypsin, 5 mg, + soyabean trypsin inhibitor, 10 mg, incubated 10' 37°C
4) goose pancreas, 5 mg
5) goose pancreas, 5 mg, + soyabean trypsin inhibitor, 10 mg, incubated 10' 37°C

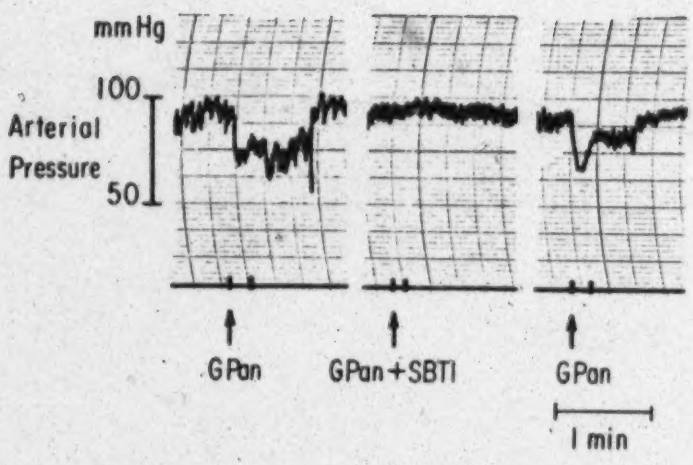


Figure 9. Bioassay of goose pancreas extract on goose blood pressure.

1) goose pancreas, 2.5 mg
2) goose pancreas, 2.5 mg, + soyabean trypsin inhibitor, 5 mg, incubated 10' 37°C
3) goose pancreas, 2.5 mg

as the previous assay, i.e., 1:2, extract to inhibitor. In this same assay a similar concentration of trypsin was blocked by the inhibitor.

Biochemical Results: The compiled results of different pancreas extracts are presented in Table III. As with the goose salt gland, an assay of the acidified, undialysed aqueous extract (method 3a) yielded negative results. When the extract was prepared as indicated in method 3b - acidified, dialysed aqueous extract - kallikrein activity was found in the unacidified portion (Goose II). This finding seemed to indicate that acidification of the extract was not important in activating kallikrein activity. Of the extracts tested, the 75% acetone preparation (method 4) was most active. Goose I extract (method 2), a six-month old preparation when tested, showed a modicum of kallikrein activity. Since no protein assay had been done, an adequate comparison with Goose III was not possible.

Although only a few biochemical assays were done, there was indication of ornithokallikrein activity. Acetone extracts appeared more active than aqueous extracts and when tested biologically (goose and hen blood pressure), vasodilatation occurred. As expected, an assay using the guinea-pig ileum was negative. Equivocal results were obtained when the assay was performed using the goose ileum. In the biological assays, the use of SBTI gave different results; in some instances it blocked all activity of the extract, and at other times there was no inhibition. When this inhibitor was used in the biochemical assays, only the trypsin activity was blocked.

Table III. Goose pancreatic estrolytic (BAEe) activity as found in aqueous and/or acetone extracts.

Sample	Specific Activity KU/mg protein	KU/g fresh tissue
Goose I (acetone extract)	no protein assay	.38
Goose II (aqueous extract - acidified)	-	-
Goose II (aqueous extract -nnon-acidified)	.0006	.07
Goose III (75% acetone preparation)	.06	.355

- = no activity

Hen Pancreas:

Biological Results: Since the original work on ornithokallikrein had been done using the hen pancreas (Werle & Hurter, 1936; Werle et al., 1966), it was decided that preparation of a suitable extract would provide a useful check on the assay techniques which had been employed and would also serve as a basis for comparison with the goose extracts.

Hen and goose pancreatic extracts (method 4), tested on one hen and one goose (Figure 10A) showed that hen extract is more active than goose extract.

A slight vasodilatation was induced by as little as 100 µg of hen pancreas when tested on hen blood pressure and 200 µg of the same preparation gave a larger and more lasting effect than 500 µg of goose pancreas. Similar concentrations of both extracts tested on goose blood pressure (Figure 10B) indicated that hen pancreas evoked a larger and slightly more prolonged vasodilatation than did goose pancreas.

The inhibitor, SBTI, was again employed to separate trypsin and ornithokallikrein activity. In tests on one hen and one goose (Figure 11), the inhibitor blocked the action of the hen pancreatic extract on both hen and goose blood pressure. As noted in some of the tests with goose pancreatic extract and SBTI, this finding is contrary to what is known about the action of this inhibitor and ornithokallikrein (Werle et al., 1966).

Attempts to use the isolated hen ileum to measure ornithokallikrein released from a suitable substrate by either goose or hen pancreatic extracts were unsuccessful.

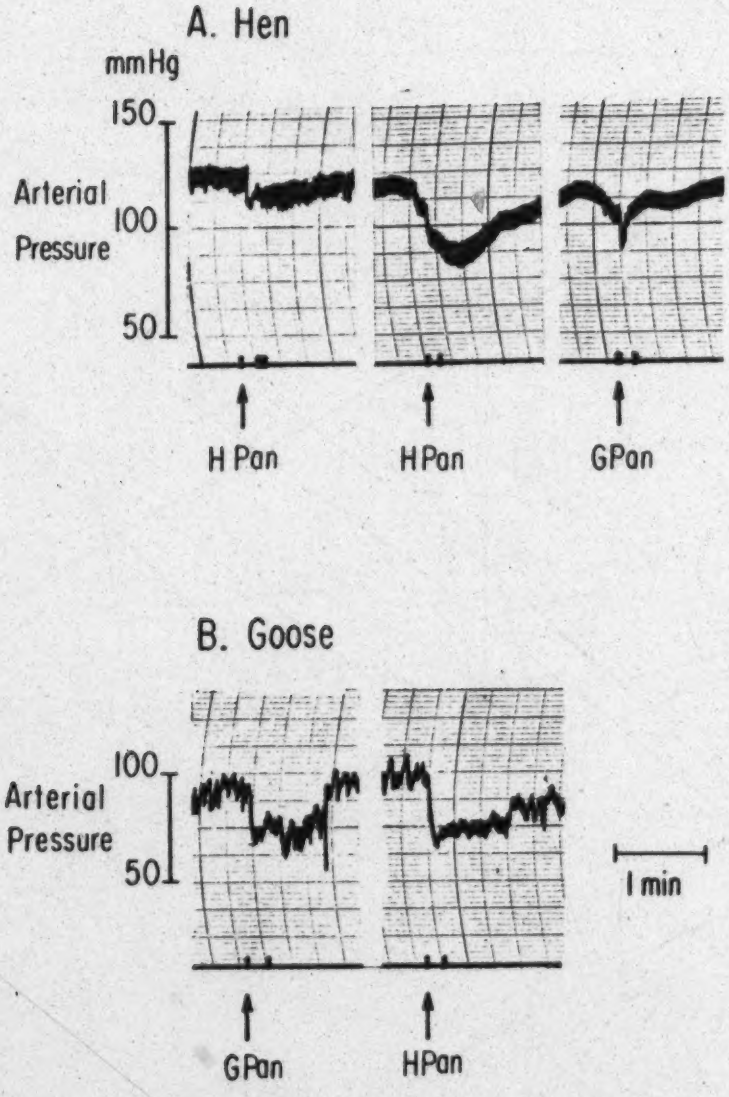


Figure 10. Bioassay of hen and goose pancreas extracts using hen and goose blood pressure.

A. 1) hen pancreas, 100 µg
   2) hen pancreas, 200 µg
   3) goose pancreas, 500 µg

B. 1) goose pancreas, 2.5 mg
   2) hen pancreas, 2.5 mg

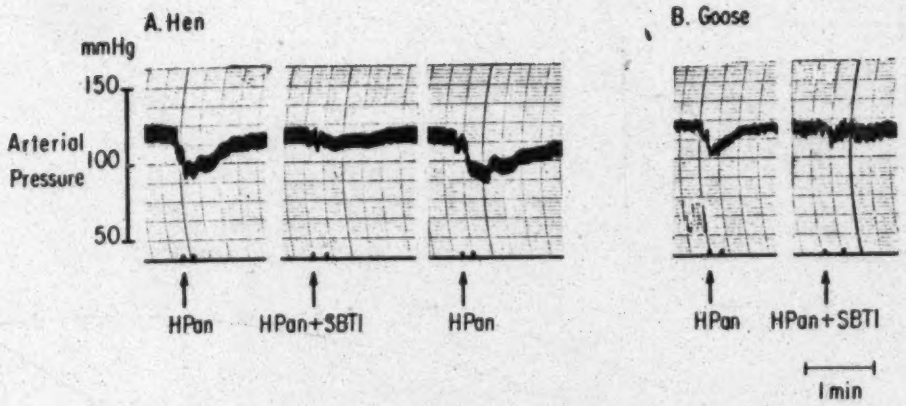


Figure 11. Bioassay of hen pancreas on hen and goose blood pressure.

A. 1) hen pancreas, 200 µg
2) hen pancreas, 200 µg, + soyabean trypsin inhibitor, 200 µg, incubated 10' 37°C
3) hen pancreas, 200 µg

B. 1) hen pancreas, 2.5 mg
2) hen pancreas, 2.5 mg, + soyabean trypsin inhibitor, 2.5 mg, incubated 10' 37°C

Biochemical Results: A number of assays (Table IV) of hen pancreatic extracts yielded interesting results. In all tests except one where both aqueous and acetone extracts of the same preparation were assayed, it appeared that the acetone extracts showed more kallikrein activity. Different centrifugation speeds, 10,000 and 13,000 rpm, of aqueous extracts were tried to see if this parameter would alter the general pattern. Results obtained were consistent with the exception noted above.

The results obtained from Hens I and II are incomplete in that assays were not done on the acetone extracts of I and the aqueous extract of II. However, the results obtained in II are consistent with those obtained for other acetone extracts. The very high specific activity found for the two tests of Hen I, acidified and non-acidified preparations, was attributable to dialysis of the aqueous homogenate which was introduced when early hen extracts were prepared. Although this particular method was followed in subsequent preparations, this high specific activity was not duplicated.

The general pattern of activity for the extracts showed that in aqueous preparations of hen pancreas, the rate of activity without any inhibitor (Figure 13) was faster than when inhibitors were present. The one exception was Hen III(a) in which the rate of activity without any inhibitor was the slowest rate. Aqueous preparations of goose pancreas followed the general pattern.

A study of the rate of activity of the acetone preparations showed an interesting result. In all hen pancreatic extracts tested except III(b), the rate of activity with SBTI was the fastest (Figure 14). The one exception followed the pattern for aqueous extracts.

Table IV. Hen pancreatic esterolytic (BAEe) activity as found in aqueous and/or acetone extracts.

Sample	Specific Activity KU/mg protein	KU/g fresh tissue
Hen I (aqueous extract - acidified)	10.4	35.2
Hen I (aqueous extract - unacidified)	8.27	39.9
Hen II (acetone extract)	.382	2.9
Hen III(a)		
aqueous extract	.524	9.6
acetone extract	.446	2.95
Hen III(b)		
aqueous extract	.298	6.3
acetone extract	2.46	12.4
Hen IV(a)		
aqueous extract	.005	.95
acetone extract	.374	3.9
Hen IV(b)		
aqueous extract	.007	.77
acetone extract	.438	2.2
Hen V		
aqueous extract	.474	21.8
acetone extract	5.4	33.9

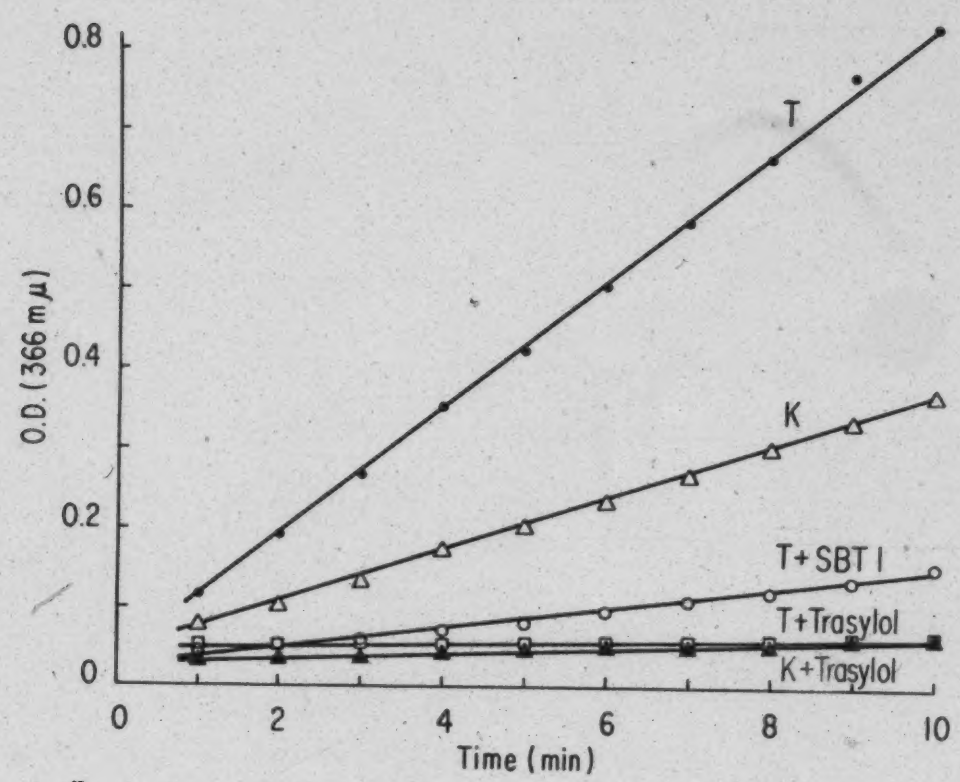


Figure 12. Inhibition of standard enzymes.

Trypsin, T, 0.8 µg (●)
Standard Kallikrein, K, 5.0 µg (△)
Trypsin, T, 0.160 µg, + soyabean trypsin inhibitor, SBTI, 0.6 µg (○)
Standard Kallikrein, K, 2.0 µg, + Trasylol, 5 KIU (▲)
Trypsin, T, 0.160 µg, + Trasylol, 5 KIU (□)

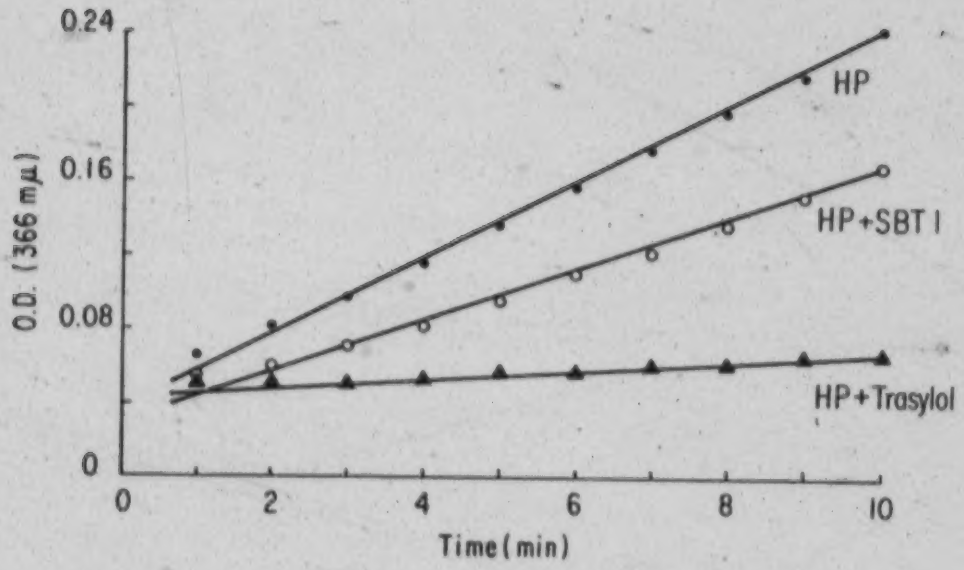


Figure 13. Hen pancreatic esterolytic (BAEe) activity of aqueous extract.

Total esterase activity, HP, 0.5 mg (●)
Hen pancreas, HP, 0.1 mg, + soyabean trypsin inhibitor, SBTI, 10 μg (○)
Hen pancreas, HP, 0.1 mg, + Trasylol, 25 KIU (▲)

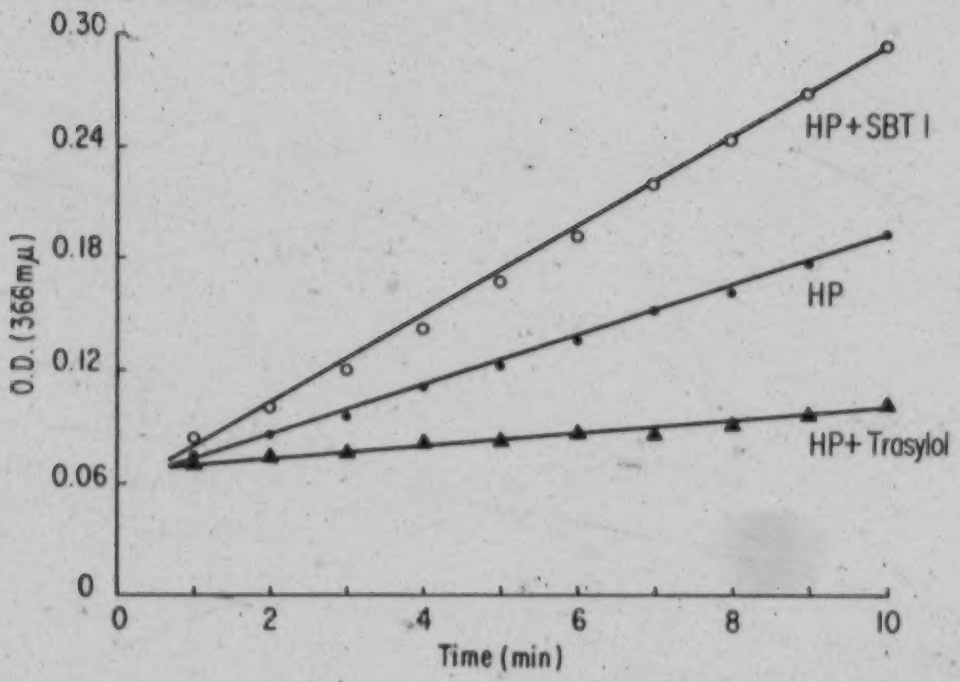


Figure 14. Hen pancreatic esterolytic (BAEe) activity of acetone extract.

Total esterase activity, HP, 10 μg (●)
Hen pancreas, HP, 2.0 μg, + soyabean trypsin inhibitor, SBTI, 10 μg (o)
Hen pancreas, HP, 2.0 μg, + Trasylol, 25 KIU (▲)

In the two geese pancreatic extracts, one showed the faster rate with and one showed the faster rate without SBTI. This pattern is similar to the results obtained in the one assay performed on acetone extract of the salt gland.

In general, the results indicated that acetone preparations seemed more active than aqueous preparations, even though there was a range of activities in the various parameters calculated. This variation could possibly be due to individual differences. All acetone extracts when tested biologically (hen and goose blood pressure) caused vasodilatation. Hen pancreatic extract was more active than goose pancreatic extract when tested in both hen and goose. The use of SBTI in the biological assays blocked all pancreatic activity, whereas only the trypsin activity was blocked in the biochemical assays.

Histological Results

A partial picture of the general organization of a secretory lobule is seen in Figure 15. One of the more prominent features is the interlobular connective tissue. The lobule is comprised of secretory cells, arranged in a radial pattern around the lumen of a secretory tubule.

A higher magnification of the secretory cells is seen in Figure 16. A better idea of the organization of the secretory cells may be noted and also the presence of intralobular connective tissue. There does not appear to be any differentiation of cell types present in the lobule.

Figure 15. Histological section of a secretory lobule of a non-functional goose salt gland. Interlobular connective tissue (ICT); secretory cells (SC). (x 40).

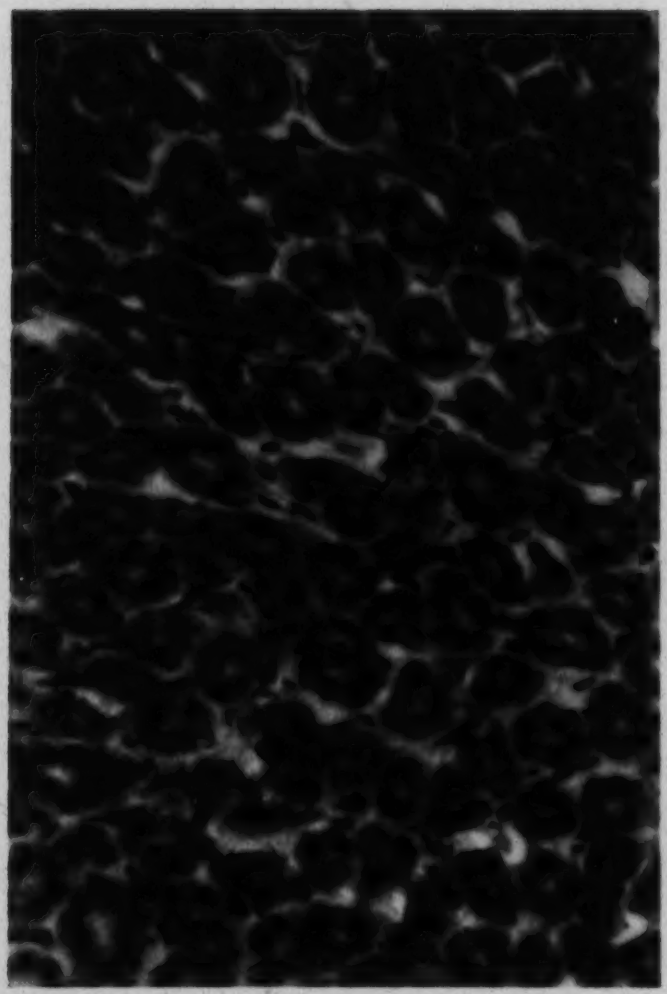


Figure 16. Histological section of secretory cells of a non-functional goose salt gland. Secretory cells (SC). (x 160).

IV. DISCUSSION

Pancreatic Extracts:

The present investigation of ornithokallikrein in the goose and hen pancreas has shown it to be activated by acetone. Acidification of tissue did not appear to be an important procedure. All extracts, both goose and hen, prepared by procedures outlined in the methods section were hypotensive (Figures 8, 10) when tested on goose and hen glood pressure. Goose pancreatic extract, when assayed on guinea-pig isolated smooth muscle, showed no kinin releasing activity when either an avian or mammalian substrate was used (Figure 6). Contrary to what might be expected, negative results were obtained when goose pancreas was assayed on isolated goose ileum. Although a slight trace might be perceptible (Figure 7), results were too tenuous to argue for successful assay with this system. The possibility that kininases might have inactivated released ornithokinin is a possibility, but it was hoped that suitable preparation of the substrate would have obviated this difficulty; however, pancreatic kininases might have been present.

The in vivo studies of goose and hen pancreatic extracts, and soyabean trypsin inhibitor yielded conflicting results. In agreement with previous investigators (Werle et al., 1966), one assay with goose pancreas and SBTI showed that trypsin but not kallikrein activity was blocked (Figure 8). This result was reproducible. A second assay, also on goose, showed that goose pancreatic kallikrein was blocked by SBTI (Figure 9). The only difference in the extracts tested was the mode of preparation. The extract assayed in Figure 8 was prepared by method 2 and the extract tested in Figure 9 was prepared by method 4.

Hen pancreatic extracts, also prepared by method 4, when tested in both hen and goose (Figure 11) showed an inhibition of what appeared to be kallikrein activity by SBTI. Although hen pancreatic extract, prepared according to method 2 was active biochemically (5.9 KU/g fresh tissue), no biological assay was done. It is not known whether the particular acetone preparation (method 4) is solely responsible for this variability.

Goose and hen pancreatic extracts when assayed biochemically showed kallikrein activity (Tables III, IV) with one exception; one acidified, aqueous extract of goose pancreas gave no results. However, an acidified preparation of hen pancreas did show kallikrein activity. Whether this is an indication of interspecies difference is not known because only one such assay was done.

A general pattern emerging from the biological and biochemical assays shows that hen pancreatic extracts are more active than goose extracts (Figure 10; KU/g fresh tissue of Tables III and IV). Since no comparative study has been done on birds, it is not known which species or which tissue is the richest source of kallikrein.

One of the problems with the assay was the fact that no purified ornithokallikrein or kinin was available as a commercial preparation. As a result, quantitative results were not possible because mammalian kallikreins and kinins have no effect in birds (Figure 4). A threshold dosage of goose pancreatic extract was not ascertained with certainty because different geese responded to a range of extracts, 250 µg to 5.0 mg. This response did not seem to be dependent on the mode of preparation. Hens were generally responsive to 100 µg of hen pancreas but a larger dose (2.5 mg) was required to elicit a response from goose. Inter- and intraspecies differences

might account for these findings.

Goose Salt Gland:

The brief histological study of the goose salt gland showed that its organization was similar to that described for ducks by Ernst and Ellis (1969). The non-functional gland has a large amount of connective tissue, and it is not possible to easily distinguish cell differentiation in the lobule, eg., between peripheral and secretory cells. Although not shown in either figure, the duct of the salt gland is lined with a columnar epithelium.

A number of assays, both biological and biochemical, were performed using the salt glands to determine the presence of ornitho-kallikrein. Tissues were prepared by various methods as outlined in Chapter II. An assay using the isolated guinea-pig ileum (Figure 3) yielded negative results. This finding would be in agreement with the well-known fact that avian kallikrein has no effect in mammalian systems. Further assays using the goose and hen blood pressures (Figures 4, 5) also yielded negative results. Although goose and hen pancreatic extracts in the same or smaller concentrations were active, there was no discernible salt gland activity.

Biochemical assays of salt glands, acidified aqueous preparations and non-acidified, dialysed preparations, showed no kallikrein activity. When a 75% acetone preparation was assayed, apparent kallikrein activity was evident. The trace of the activity was similar to that determined for assays of hen pancreas (75% acetone preparation), and indicated that there was an apparent stimulation of activity by adding SBTI (Figure 14).

Some studies were then undertaken to check the assay system. Although it had been reported that ornithokallikrein hydrolyses BAEe (Werle et al., 1966), the exact assay system was not indicated.

The checks carried out, using hen and goose pancreas and goose salt glands, included alteration of SBTI concentrations (0.5 mg to 5.0 mg/ml); different volumes of BAEe (50 to 500 µl); a check to see if other components of the assay showed absorbance at 366 mµ; use of .001% C_2H_5OH in the assay system instead of ADH.

The results indicated that two problems might exist in the assay when 75% acetone extracts were used: (1) absorbance by other components of assay system at 366 mµ; (2) apparent stimulation within the assay system by addition of SBTI, and SBTI + HP.

Some attempt was made to correct for the first problem on the traces obtained of enzyme activity, but this did not greatly alter the original results. The possible percentage of stimulation within the assay system was not accounted for. It seems valid to say that this particular assay system is not suitable for use with 75% acetone tissue extracts, but could be used with other extracts as already indicated. Another possible reason for these results might be the presence of residual acetone in the extracts since the only means taken to dry them was in the dessicator. The possible action of acetone on the assay system was not tested.

In spite of these problems, the biological and biochemical assays show that ornithokallikrein is present in the goose and hen pancreatic extracts, but there does not seem to any in the salt gland. There is still the possibility that the methods used failed to extract

it from the gland or that it requires a very different activating system from those already known for other kallikreins.

The evidence obtained in these experiments makes it extremely unlikely that a kallikrein-like enzyme plays a part in physiological vasodilatation in the salt gland of the goose. The vasodilatation which accompanies secretory nerve stimulation in this gland, like that of the salivary glands of mammals is atropine resistant, hence there would appear to be a parallel between these two different systems in birds and mammals. The fact that, unlike the submaxillary glands of mammals, the salt gland of the goose, and probably of other similar birds, contains no kallikrein-like substance would appear to make it unlikely that submaxillary kallikrein is the mediator of vasodilatation in the salivary gland of mammals. The atropine-resistant vasodilator effects of parasympathetic nerve stimulation in birds and mammals remains an interesting and unexplained phenomenon.

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